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Comparing Eco-Friendly Biochrome - Prodigiosin Yield in *Serratia marcescens* and *Serratia rubidaea* using OFAT Approach

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ABSTRACT

Microbial biochromes are aesthetically alluring secondary metabolites that also display multifaceted applications. Prodigiosin is one such, pink to red, environmentally safe, biochrome produced by certain species of genera *Serratia*, *Pseudomonas* and *Vibrio*. It has found applications in several industries from textile to therapeutics. Currently, as per consumer demand for natural products, replacing synthetic pigments with biochromes like Prodigiosin can lead to several sustainable and long-term environmental benefits. However, Prodigiosin production faces concerns about economic viability and low yields. Moreover, most optimization studies focus on *S. marcescens* strains, overlooking the resource of *S. rubidaea*, which also holds prodigiosin production candidature. Hence, the present study aims at identifying and comparing key physical and chemical parameters, leading to prodigiosin yields using the one factor at a time approach (OFAT), in previously isolated *S. marcescens* and *S. rubidaea*. Optimization of physical parameters like pH, temperature and incubation time, showed significantly higher yields at pH 6, 28°C and 96 hrs for both isolates. On comparing the chemical factors, the most significant carbon sources for *S. marcescens* and *S. rubidaea* were glycerol and glucose respectively, while yeast extract was the most effective nitrogen source for both. On studying the effect of supplements, $FeSO_4$ and proline were found to be most effective for both the isolates. Considering all the effective factors, a production medium was designed which gave a 2.61-fold and 2.44-fold increase in prodigiosin yield for *S. marcescens* and *S. rubidaea*, respectively.

Keywords Biochrome, Prodigiosin, *Serratia marcescens, Serratia rubidaea*, OFAT.

INTRODUCTION

Colors play a substantial role in human life. They create beauty, appeal, remembrance and a glossary about all products. Apart from these attributes, colors enact as the principal visual communication language across the universe. Understanding the importance of colors, and the substances that impart them do have an indispensable significance. Pigments are substances that impart colors. They have a natural and chemical/synthetic origin. The environmental and ecological adverse effects of prolonged and uncontrolled use of synthetic pigments in the food, textile, cosmetics, and pharmaceutical industry is surfacing for a decade now.

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Moreover, the gravity of the synthetic dye concerns is augmenting, as many of them are already associated with concerns like recalcitrance, toxicity, mutagenicity, allergy, carcinogenesis, and bioaccumulation. Natural pigments which are environmentally safe and non-toxic can provide an effective alternative to synthetic counterparts (Han *et al.* 2021, Lagorio 2013, Joshi *et al.* 2003, Lellis *et al.* 2019, Ardila-Leal *et al.* 2021).

Among the natural pigments, the demand and acceptance for biological pigments are augmenting across different industries owing to consumer demand and environmental logistics. Conventional plant pigments offer limited bichrome diversity, significant yield inconsistency, and environment-dependent color variability. Microbial pigments on the other hand cater to several plant pigment concerns. Moreover, the evolving fermentation technology, synthetic biology, and recombinant DNA technology provide mechanisms to increase yield consistently making microbial biochromes a preferred choice (Darshan and Manonmani 2015, Rao *et al.* 2017).

Prodigiosin is one such microbial biochrome, that has an eye-appealing color range from pink to red. Structurally, prodigiosin ($C_{20}H_{25}N_3O$) is a tripyrrole molecule. Its A, B and C ring represents pyrrole, methoxypyrrole, and 2- methyl-3-pentylpyrrole, respectively. Recent structural investigations have revealed prodigiosin can form several linear and ring derivates constituting collectively the family of prodiginines. It is produced by restricted genera of *Eubacteriales* and *Actinomycetales* and certain well-recognized bacteria producing prodigiosin include *Serratia marcescens*, *Serratia rubidaea, Alteromonas rubra, Vibrio gazogenes, Pseudomonas magnestorubra*.

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Prodigiosin has demonstrated comprehensive applications in the pharmaceutical sector like antibacterial, antifungal, antitumor, antiprotozoal, anticancer, and immunosuppressive activity. In the food sector, apart from enhancing aesthetics, it enacts as a food preservative, whereas in the textile industry it acts as a fabric dye (Giri *et al.* 2004, Ramesh *et al*. 2020, Domröse *et al.* 2015, Han *et al.* 2021, Nguyen *et al.* 2019). However, major concerns hindering its applicability are low yield and fermentation economics. Despite this shortcoming, optimization studies explore only scarce sources of prodigiosin like *S. marcescens* and *A. rubra*, and *V. gazogenes*, leaving other sources underexplored. Hence, considering its diverse and noteworthy applications, it becomes essential to identify inexpensive and yield-promoting nutritional and environmental parameters across different Prodigiosin producers.

One factor at a time approach (OFAT) though traditional, remains a promising choice for the preliminary determination of yield-promoting parameters in bacterial platforms. It's a simple easy and reliable method to identify effective variable parameters while maintaining other parameters constant (Abdel-Rahman *et al.* 2020). Thus, in the present study prodigiosin yield from *Serratia marcescens* and *Serratia rubidaea* was monitored using this approach. Once optimum parameters are determined methodologies to devise inexpensive processes can be vouched for.

MATERIALS AND METHODS

All chemicals, solvents, and bacteriological media used in the present study were of analytical and microbiological grade. They were procured from Himedia. For the preparation of media and chemicals, distilled water was used. All experiments were carried out in triplicates using borosilicate acid and alkali-resistant glassware.

Serratia marcescens and *Serratia rubidaea* previously isolated from the marine ecosystem were used in the present study. They were confirmed to produce prodigiosin by chemical tests and spectroscopic analysis. The cultures were maintained at 4°C on sterile nutrient agar slants throughout the study. For inoculation, in the case of each media standardized suspension of *S. marcescens* and *S. rubidaea* was freshly prepared. Prodigiosin yield was monitored for each parameter using the OFAT approach (Abdel-Rahman *et al.* 2020).

Preparation of standard graph

Inoculation was done on a sterile nutrient agar medium. Following inoculation, incubation was carried out for 48 hrs at room temperature. Post-cultivation

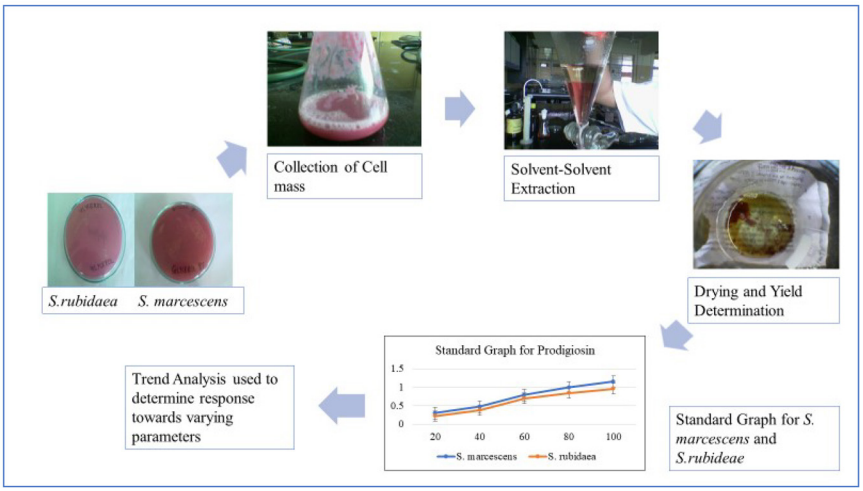

Fig. 1. Methodology for prodigiosin yield determination in *S. marcescens* and *S. rubidaea*.

the cell biomass was collected, and wet weight was determined. The cell biomass was processed for prodigiosin extraction using acetone at a ratio of 1:10. The prodigiosin supernatant, was separated by centrifugation, vacuum dried, and weighted. Different concentrations of extract were used to prepare a standard graph by recording the absorbance at 530 nm (absorption maxima for prodigiosin as determined previously). The yields in the case of each factor under consideration were then determined using trend analysis for both isolates (Fig. 1).

Physical parameters

Effect of pH

Inoculation was done on a sterile nutrient agar medium adjusted to pH 5, pH 6, pH 7, pH 8, and pH 9. Incubation was done at room temperature for 48hrs post-inoculation.

Effect of temperature

Inoculation was carried out on a sterile nutrient agar medium. Post-inoculation incubation was carried out at different temperatures such as 10°C, 20°C, 28°C, 37°C, and 45°C for 48 hrs.

Effect of incubation time

Inoculation was carried out on a sterile nutrient agar medium. The media were then incubated at different time intervals like 24 hrs, 48 hrs, 72 hrs and 96 hrs, and 120 hrs at room temperature.

Chemical Parameters

Effect of carbon sources

Inoculation was done on the control medium namely sterile peptone agar medium (1%). Simultaneously cultures were also inoculated on a peptone medium containing 1% of different sugars like xylose, arabinose, glucose, fructose, lactose, mannitol, sucrose, glycerol and starch. Incubation was carried out at room temperature for 48 hrs post-inoculation.

Effect of nitrogen sources

Inoculation was carried out on the control medium namely sterile glucose medium (1%). Simultaneously cultures were also inoculated on a glucose medium containing 1% of different nitrogen sources like ammonium citrate, ammonium chloride, casamino acids, casein, peptone, yeast extract, meat extract, gelatin,

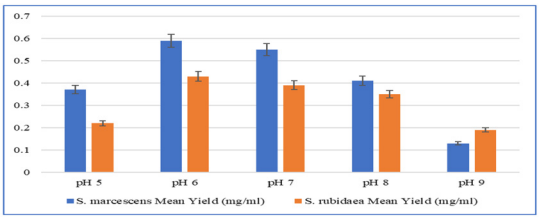

Fig. 2. Effect of pH on prodigiosin yield in *S. marcescens* and *S. rubidaea*.

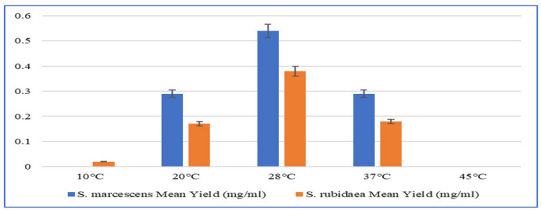

Fig. 3. Effect of temperature on prodigiosin yield in *S. marcescens* and *S. rubidaea*.

and soya bean digest. Incubation was done at room temperature for 48 hrs post-inoculation.

Effect of supplements

Inoculation was carried out on the control medium namely a sterile nutrient agar medium. Simultaneously cultures were also inoculated on a sterile nutrient agar medium containing different supplements like amino acids (1%) - proline, tyrosine, tryptophan, phenylalanine, histidine, and salts (0.1%) - NaCl, $MgSO_4$, K_2HPO_4, $FeSO_4$. Incubation was carried out at room temperature for 48 hrs post-inoculation.

Effect of production media

Inoculation was carried out on a control medium namely a sterile nutrient agar medium. Simultaneously cultures were also inoculated on production media prepared using effective physical, chemical, and supplement parameters. Prodigiosin yield from the production medium was compared to the control medium.

RESULTS AND DISCUSSION

Preparation of standard graph

For *S. marcescens* and *S. rubidaea*, the prodigiosin standard graph was prepared in the concentration range of 0.1 mg/ml to 1 mg/ml. As per the regression analysis, the R^2 value for the standard graph of both extracts was close to 1. This indicates smaller differences between observed data and goodness of fit. The standard graph was then used to predict prodigiosin yield in response to parameters under investigation using trend analysis.

Physical parameters

Effect of pH

According to Yip *et al.* (2019), prodigiosin production occurs at a pH range of 4 to 9. In the present study, a similar observation was noted. Moreover, in *S. marcescens* and *S. rubidaea* prodigiosin yield was significantly ($p<0.05$) higher at pH 6. This observation agrees with the findings of Siva *et al*. (2012), Mohammed *et al.* (2018) and Halder *et al.* (2020). This result also confirms the report of Han *et al.* (2021) who reported pH 6 to 8 as the optimum pH for prodigiosin yield in *Serratia* spp. Despite sharing the same effective pH, yield variability was noted across the species. Prodigiosin yield for *S. rubidaea* was significantly ($p<0.05$) lower than *S. marcescens* at all the investigated pH. As per Solé *et al.* (1994) pH plays an important role in the decomposition of substrates and transportation of prodigiosin, thus dictating the accumulation of prodigiosin (Fig. 2).

Effect of temperature

According to Pryce and Terry (2000), prodigiosin production takes place at a temperature range of 22°C to 30°C. Moreover, Romanowski *et al.* (2019), mention's transcription of prodigiosin biosynthetic operon decreases with an increase in temperature above 30°C. In the present study, prodigiosin yield from *S. marcescens* and *S. rubidaea* was recorded over a temperature range from 10°C to 37°C endorsing the high-temperature sensitivity. For both the isolates, the yield was significantly ($p<0.05$) higher at 28°C. This result agrees with the findings of Bhagwat and Padalia (2020) and Siva *et al.* (2012). Furthermore, like the effect of pH, despite sharing the same effective temperature, prodigiosin yield from *S. rubidaea*

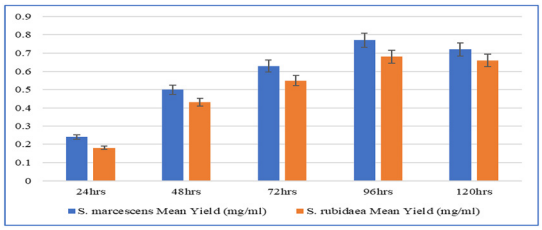

Fig. 4. Effect of incubation time on prodigiosin yield in *S. marcescens* and *S. rubidaea*.

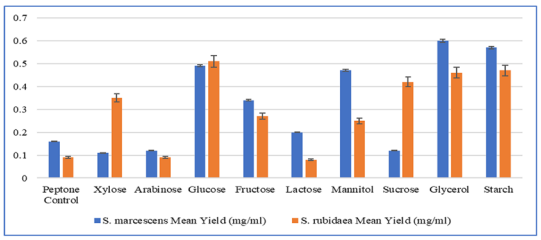

Fig. 5. Effect of carbon sources on prodigiosin yield in *S. marcescens* and *S. rubidaea*.

was significantly ($p<0.05$) lower than *S. marcescens* at all the investigated temperatures. For both the isolates, no yields could be recorded at 45°C which confirms the reports of Qadri and Williams (1972). The high-temperature sensitivity towards prodigiosin production has also been traced down to the last condensation step carried out by Pig C, which is denatured at temperatures above 30°C. Despite this, in the present study yields were recorded at 37°C. This observation, therefore, contradicts the findings of Giri *et al.* (2004) who reported no prodigiosin yield on nutrient medium at 37°C for *S. marcescens* (Fig. 3).

Effect of incubation time

Extended incubation periods are fruitful for prodigiosin yield as per Elkenawy *et al*. (2017) and Xu *et al.* (2011). In the current study also, prodigiosin yield for both the isolates was found to increase with an increase in incubation time of up to 96 hrs. For *S. marcescens* and *S. rubidaea* prodigiosin yield was significantly higher at 96 hrs. In concurrence, Srimathi *et al.* (2017) reported 96hours for significant prodigiosin production in *Serratia marcescens*. To add on, alike to the effect of pH and temperature, despite sharing the same effective incubation time, prodigiosin yield from *S. rubidaea* was significantly ($p<0.05$) lower than *S. marcescens* at all the investigated periods. According to Bhagwat and Padalia (2020), longer incubation time could be co-related to the fact that Prodigiosin is a secondary metabolite that may display a stress-protective role (Fig. 4).

Chemical parameters

Effect of carbon sources

Prodigiosin yield for *S. marcescens* in the control medium increased significantly ($p<0.05$) with the addition of carbon sources except for xylose, arabinose, and sucrose. Correspondingly, for *S. rubidaea* also prodigiosin yield in the control medium increased significantly ($p<0.05$) with the addition of carbon sources except for arabinose and sucrose.

Table 1. Effect of production media on prodigiosin yield in *S. marcescens* and *S. rubidaea*.

	Control medium Nutrient agar *S. marcescens*	Control medium Nutrient agar *S. rubidaea*	Production media *S. marcescens*	Production media *S. rubidaea*
Carbon source	Meat extract and peptone	Meat extract and peptone	1% Glycerol	1% Glucose
Nitrogen source	Meat extract and peptone	Meat extract and peptone	1% yeast extract	1% yeast extract
Supplements	Nil	Nil	1% proline, 0.1% $FeSO_4$	1% proline, 0.1% $FeSO_4$
Temperature	28°C	28°C	28°C	28°C
pH	7	7	6	6
Incubation time	48 hrs	48 hrs	96 hrs	96 hrs
Yield	0.57±0.71 mg/ml	0.45±0.31 mg/ml	1.49±0.63 mg/ml	1.1±0.22 mg/ml

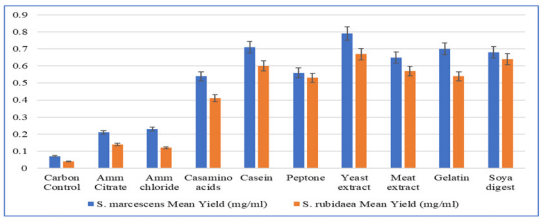

Fig. 6. Effect of nitrogen sources on prodigiosin yield in *S. marcescens* and *S. rubidaea.*

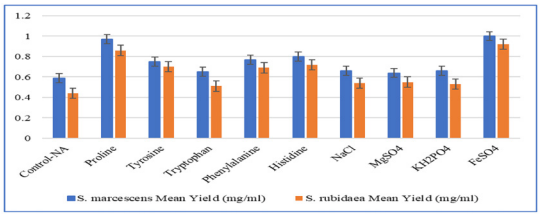

Fig. 7. Effect of supplements on prodigiosin yield in *S. marcescens* and *S. rubidaea.*

Furthermore, glycerol and glucose were found to be most effective for *S. marcescens* and *S. rubidaea,* respectively. This observation is concurrent with the findings of Giri *et al.* (2004) who reported prominent prodigiosin yield in nutrient broth and glycerol broth. Chang *et al.* (2011) and Lin *et al.* (2019) also emphasized the importance of glycerol and glucose in enhancing prodigiosin yield. However, similar to physical factors, prodigiosin yields for *S. rubidaea* were significantly ($p<0.05$) lower than *S. marcescens* in presence of all the carbon sources except xylose and glucose (Fig. 5).

Effect of nitrogen sources

Prodigiosin yield for *S. marcescens* and *S. rubidaea* in the control medium increased significantly ($p<0.05$) with the addition of all nitrogen sources. Yields were substantially higher for all complex nitrogen sources especially yeast extract, casein, and gelatin. This observation is concurrent with the findings of Tao *et al.* (2005) who reported the significance of yeast extract and peptone in prodigiosin production. Lin *et al.* (2019) also mentioned the importance of complex nitrogen sources in enhancing prodigiosin yield. However, despite sharing the same inclination towards the complex nitrogen sources, prodigiosin yields for *S. rubidaea* were significantly ($p<0.05$) lower than *S. marcescens* in presence of all the nitrogen sources (Fig. 6).

Effect of supplements

Prodigiosin yield for *S. marcescens* and *S. rubidaea* in the control medium increased significantly ($p<0.05$) with the addition of all supplements. On comparing, the amino acid supplements – proline and histidine were highly effective, while, in the case of salts $FeSO_4$ was most effective. Analogous to the present observation, Pan *et al.* (2019) and Wei *et al.* (2005) reported proline supplementation to enhance prodigiosin yield. $FeSO_4$ as a prodigiosin yield enhancer was also noticed by Liang *et al.* (2013). Despite expressing a similar response towards supplements, prodigiosin yields in *S. marcescens* were significantly ($p<0.05$) higher than in *S. rubidaea* (Fig. 7).

Effect of production media

Designed production media (Table 1) led to a 2.61-fold and 2.44-fold increase in prodigiosin yield for *S. marcescens* and *S. rubidaea*, respectively. Co-linear to the present study, for *S. marcescens* strains, Faraag *et al.* (2017) and Wang *et al.* (2012) reported 1.96-fold and a 2-fold increase in yield, respectively, while for *S. rubidaea* strains a 2.6-fold increase in yield was reported by Siva *et al.* (2012). Furthermore, being consistent with previous observations prodigiosin yield for *S. marcescens* was significantly ($p<0.05$) higher than *S. rubidaea*. Considering the suitable nutritional factors, corresponding cheap agricultural/industrial byproducts can now be employed to make the process economical (Table 1).

CONCLUSION

Exploring and determining eco-friendly, prodigiosin yield-promoting parameters in all known resources are required to make the production economically viable. In the present study, the OFAT approach revealed key physical and chemical parameters in scarcely investigated *S. rubidaea*. Prodigiosin yields under optimized conditions for *S. rubidaea* (1.1±0.22mg/ml) were only marginally lower than *S.*

marcescens (1.49±0.63 mg/ml) and hence hold equal candidature for economical prodigiosin production. Further studies will focus on cheap waste by-products of several industries to make prodigiosin production in *S. rubidaea* economical. The prodigiosin thus produced can be employed to replace synthetic counterparts in food, textile, and cosmetic industries.

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